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Award Number: W81XWH-04-1-0789

TITLE: Critical Importance of Protein 4.1 in Centrosome and Mitotic Spindle Aberrations
in Breast Cancer Pathogenesis

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REPORT DATE: September 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
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1. REPORT DATE 01-09-2006		2. REPORT TYPE Final		3. DATES COVERED 1 Sep 2004 – 31 Aug 2006	
4. TITLE AND SUBTITLE Critical Importance of Protein 4.1 in Centrosome and Mitotic Spindle Aberrations in Breast Cancer Pathogenesis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0789	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sharon W. Krauss, Ph. D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Lawrence Berkeley National Laboratory Berkeley, CA 94720				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT <p>We proposed to test the novel hypothesis that protein 4.1 is of critical importance to centrosome and mitotic spindle aberrations that directly impact aspects of breast cancer pathogenesis. We characterized 4.1R, one member of the 4.1 family, as a component of mature centrosomes, major microtubule organizing structures in interphasic cells. Mature centrosomes become poles of mitotic spindles responsible for accurate segregation of duplicated chromosomes between dividing cells. We reasoned that aberrant 4.1 expression could engender defects in functions of centrosomes, mitotic spindles and in cytokinesis. We first analyzed centrosomal distributions of 4.1R in breast cancer cell lines with normal vs. hyper-amplified centrosomes. We observed that 4.1R resides at only a subset of amplified centrosomes in the malignant breast cancer cell lines. Thus the hyperamplified centrosomes are not fully mature. We next directly tested effects of downregulating 4.1R expression. We identified specific RNA duplexes which silence 4.1R (RNAi). After exposing human cells to 4.1R RNAi, we characterized perturbed centrosomal functions, several classes of aberrant mitotic spindles, defects in cytokinesis and altered cell cycle progression. Thus our initial data strongly support our hypothesis. If funding can be obtained for future investigations of 4.1 in breast cancer cell lines and tissue samples, prognostic and diagnostic tests based on assessing genetic variations as well as levels of expression of this gene in individuals could be implemented. These may be as straightforward as analysis of lymphocytes isolated from patient blood samples (4.1R is expressed in lymphocytes). An understanding of the roles of 4.1 in centrosomal and spindle abnormalities characteristic of many breast cancers can also lead to identification of 4.1 or other interacting proteins as new therapeutic targets.</p>					
15. SUBJECT TERMS protein 4.1, hyperamplified centrosomes, mitotic spindles, cytokinesis					
16. SECURITY CLASSIFICATION OF:			UU	18. NUMBER OF PAGES 14	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 10

Reportable Outcomes..... 10

Conclusions..... 11

References..... 12

Appendices..... 12

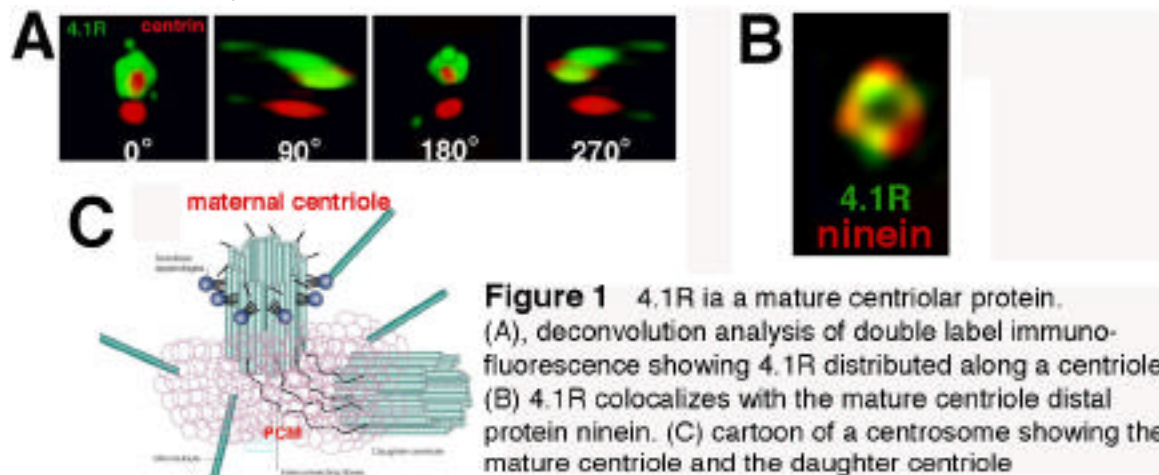
INTRODUCTION

Breast cancer biogenesis is hypothesized to be a multi-step process and pathologically many breast cancers are characterized by cell division defects such as amplified (supernumerary) centrosomes, mitotic spindle pole defects responsible for aberrant chromosome segregation, and perturbed cytokinesis. We previously established that protein 4.1R, formerly characterized solely as a crucial membrane skeletal protein in human red cells, is an essential component of centrosomes, mitotic spindles and localizes at the midbody during cytokinesis (Krauss et al., 1997b; Krauss et al., 1997a; Krauss et al., 2004). Centrosomes are the microtubule organizing centers of cells (microtubules are structures responsible for cellular architecture, motility, polarity, and intracellular transport). Centrosomes become the poles of mitotic spindles which partition chromosomes during mitosis and centrosomes also regulate final abscission stages of cytokinesis. Based on our investigations, we proposed to test the hypothesis that aberrant 4.1 expression could adversely affect centrosome and spindle structure/function, perturb cytokinesis and induce genetic instability during breast cancer biogenesis.

BODY

Task 1: Assess 4.1 distribution in amplified centrosomes and in spindle poles of breast cancer cell lines with amplified centrosomes/abnormal mitotic spindles.

Although protein 4.1 was previously identified as a multifunctional structural protein in red cells, protein 4.1 has very recently been identified as a family of proteins: 4.1R, 4.1G, 4.1B and 4.1N. Due to this unanticipated complication, it was important to first characterize “4.1” antibodies as to their reactivity with different family members. Then using a gene-specific antibody against 4.1R, we needed to precisely characterize 4.1R (red cell) at centrosomes since detailed localization of 4.1R within centrosomal regions provides an important clue as to its potential functions in breast cancer cells. We succeed in determining that 4.1R is a mature (maternal) centriole component. More specifically, we determined that 4.1R resides at the distal region of mature centrioles, an area which anchors microtubules.



Based on our new observation that 4.1R is a protein component of mature centrioles, it would be expected to associate with a second maturing centriole during completion of S phase in cells with normal numbers of centrosomes. However, how it would be distributed in breast

cancer cells with hyperamplified centrosomes was a goal of this Task. Additionally, assessing its distribution in normal cells and in breast cancer cells was relevant to Task 3 to determine if 4.1R localization at mature centrioles changed during cell cycle progression. Therefore we initially analyzed distributions of 4.1R epitopes by double label immunofluorescence in synchronized diploid human fibroblasts using the microtubule anchoring protein ninein as a mature centriole marker. 4.1R co-localized with ninein at a single centriole in a ring-shaped distribution in G1, began to extend to a second ninein-labeled maturing centriole during S phase (“one and a half rings”) and colocalized with ninein at both mature centrioles (two ring pattern) during G2.

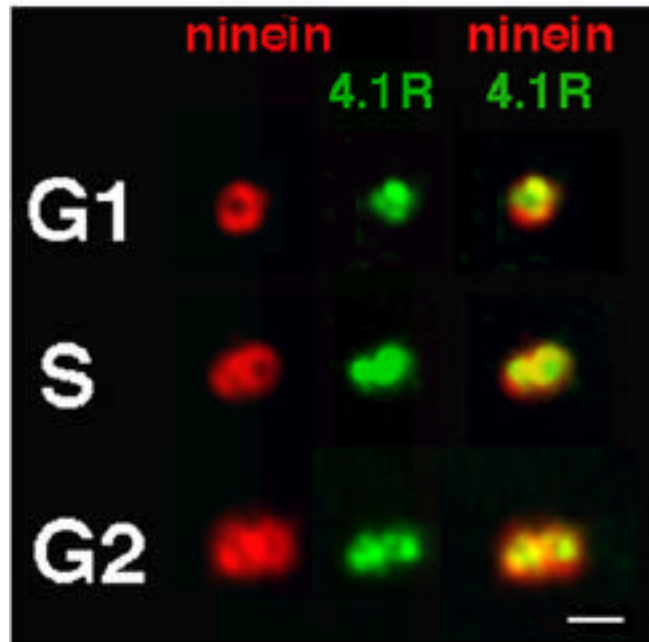


Figure 2 Distribution of 4.1R during cell cycle progression. Human fibroblasts were synchronized using double thymidine block. By indirect immunofluorescence, 4.1R localizes exclusively to centrosomes also containing ninein. bar=0.5 μ .

We applied our findings to analyze 4.1R centrosomal distribution in breast cancer cell lines. Initially we had to make modifications of fixation, permeabilization and immunolabeling conditions to ensure imaging of complete sets of centrosomes with various antibody probes in the breast cancer cell lines. We used a well-established centrosomal marker, anti-centrin, in double label experiments with antibody against 4.1R. In breast cancer cells such as MCF10A with normal centrosome numbers, we determined that 4.1R localizes to one or both centrosomes in randomly growing populations (Figure 3). This pattern was identical to that we observed in normal diploid human cells (Figure 2). By contrast, in breast cancer cell lines with amplified centrosomes (MDAMB231 and T47D) detected by centrin labeling, we observed 4.1R epitopes at a subset of hyperamplified centrioles (Figure 3; arrows show examples of centrioles which do not have associated 4.1R).

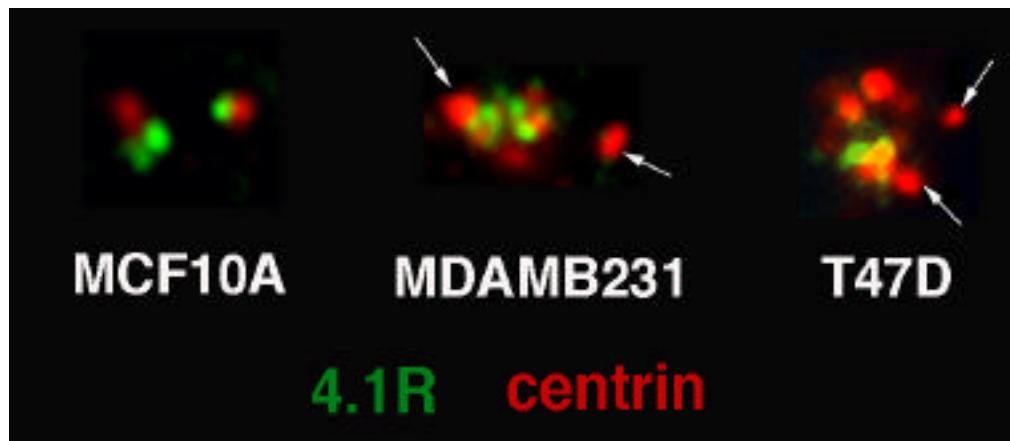


Figure 3 Protein 4.1R is present at a subset of centrosomes in breast cancer cell lines with hyperamplified centrosomes. Double label immunofluorescence was performed on breast cancer cell lines probing for centrin to label centrosomes and 4.1R epitopes. In MCF10A, a breast cancer cell line with normal centrosomes, 4.1R epitopes are present at one or both centrosomes (an example of 4.1R at both centrosomes is shown). In two aggressive breast cancer tumor cell lines with hyperamplified centrosomes as detected by multiple centrin-stained centrosomes, 4.1R is present at a subset of centrosomes (arrows indicate centrosomes with no associated 4.1R).

These sets of observations provide an important clue as to molecular mechanisms generating hyperamplification in these aggressive breast cancer cells. Hyperamplified (supernumerary) centrosomes are associated with poor clinical outcomes and amplification can result from unregulated centrosome duplication during S phase of the cell cycle (i.e. several rounds of centrosome duplication within the same cell cycle), mitotic failure, tetraploidization (e.g. cell fusion) and/or cytokinesis failure (Nigg, 2002). Because 4.1R is present at only a subset of hyperamplified centrosomes in breast cancer cells, our preliminary results suggest that at least one mechanism for amplification is unregulated centrosome duplication because centrosomes have reduplicated but have not sufficiently matured (which occurs at G2 of the cell cycle) to acquire 4.1R. Due to the unanticipated but crucial need to initially fully characterize the subcentrosomal distribution of 4.1R, plus additional modifications needed for optimization of imaging according to individual antibodies and cell lines within the time frame and budget of this Concept Award, we were not able to fully extend our experiments to all the breast cancer lines originally proposed with all the antibody probe combinations. However, the powerful preliminary data presented above have important implications for breast cancer tissue architecture and chromosome segregation since immature centrosomes have compromised capacities to anchor and organize microtubules. Thus if we can successfully obtain further funding to extensively assess the distribution of 4.1R, along with other mature centrosome markers, in even more breast cancer cell lines as well as in breast cancer tissue, we potentially can experimentally determine mechanisms and contributions of 4.1R to the biogenesis of centrosome amplification in various types and stages breast cancer.

Task 2: Use RNAi to downregulate 4.1 expression in breast cancer cell lines with a range of amplified or unamplified centrosomes by targeting highly conserved 4.1 domains or unique 4.1 domains.

We have successfully identified three RNAi duplexes and established transfection conditions which downregulate 4.1R expression in model transformed human epithelial cells. Each duplex

individually depletes 4.1R, an important observation to rule out off-target effects. Additionally, the duplexes do not downregulate 4.1G expression, a ubiquitous and newly identified 4.1 family member with some highly homologous regions which is generally expressed in most cells. We have also identified a variant related RNA duplex which does not affect 4.1 1R expression which is a crucial control for our experiments.

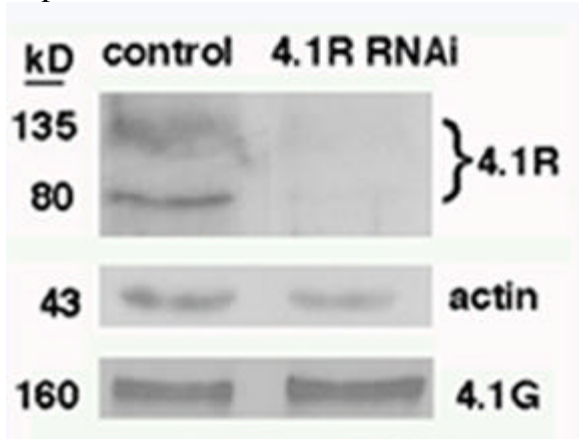


Figure 4 Downregulation of 4.1R expression after RNAi. Representative Western blot of 4.1R and 4.1G after exposure for 96 hrs with 4.1R RNAi. Expression of 4.1R decreased 70-85% in HeLa cells and 50-70% in CaSki cells when normalized to actin as a loading control while 4.1G expression was not markedly altered.

Given these data, we are now poised to apply this powerful RNAi technology to downregulate 4.1R expression directly and specifically in breast cancer cell lines.

Task 3: Analyze morphology, cell cycling and pathologic consequences of 4.1 downregulation in breast cancer cell lines.

To identify key morphological and pathological parameters when 4.1R function is altered which are relevant to breast cancer, RNAi-mediated protein depletion experiments were first performed in model human cells which have better established conditions for imaging. Tubulin immunostaining revealed 31% of 4.1R-depleted cells with disorganized whorls of cytoplasmic microtubules, in contrast to well-organized radial arrays in 98% of control cells.

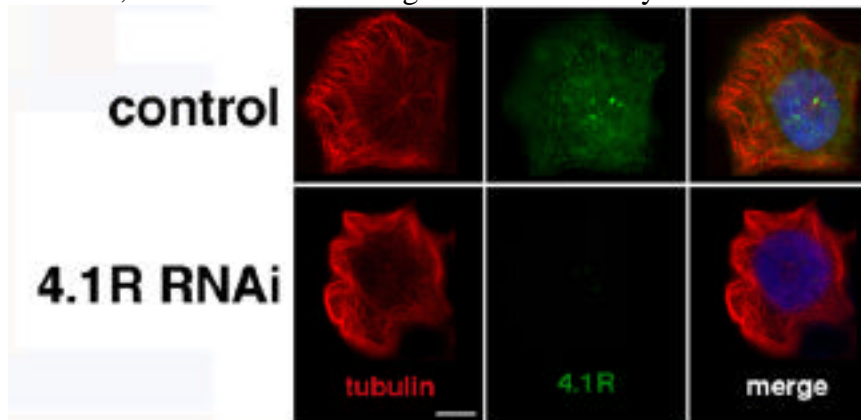


Figure 5 Effects of downregulation of 4.1R expression on microtubules. Immunofluorescent staining of tubulin and 4.1R. In 4.1R RNAi-treated cells with no detectable 4.1R signals over background, microtubules appear whorled in contrast to controls with microtubules radiating from a focal point (arrow). Bar=10 μ .

This experiment shows that depletion of 4.1R from centrosomes compromises their functional capacity to anchor and organize microtubules in interphase.

Centrosomes become the poles of mitotic spindles, the cellular apparatus responsible for the accurate distribution of chromosomes. Since we observed that centrosomes with depleted 4.1R have compromised functions such as tubulin organization during interphase, we examined mitotic spindles assembled during 4.1R silencing by RNAi. By double label immunofluorescence, we determined that specific downregulation of 4.1R resulted in three major classes of mitotic spindle defects: monopolar, multipolar and bipolar spindles with decondensed, misaligned chromatin. These classes of defective mitotic spindles are “markers” of cancer cells, have been observed in breast cancers, and are highly predictive of chromosome instability and aneuploidy.

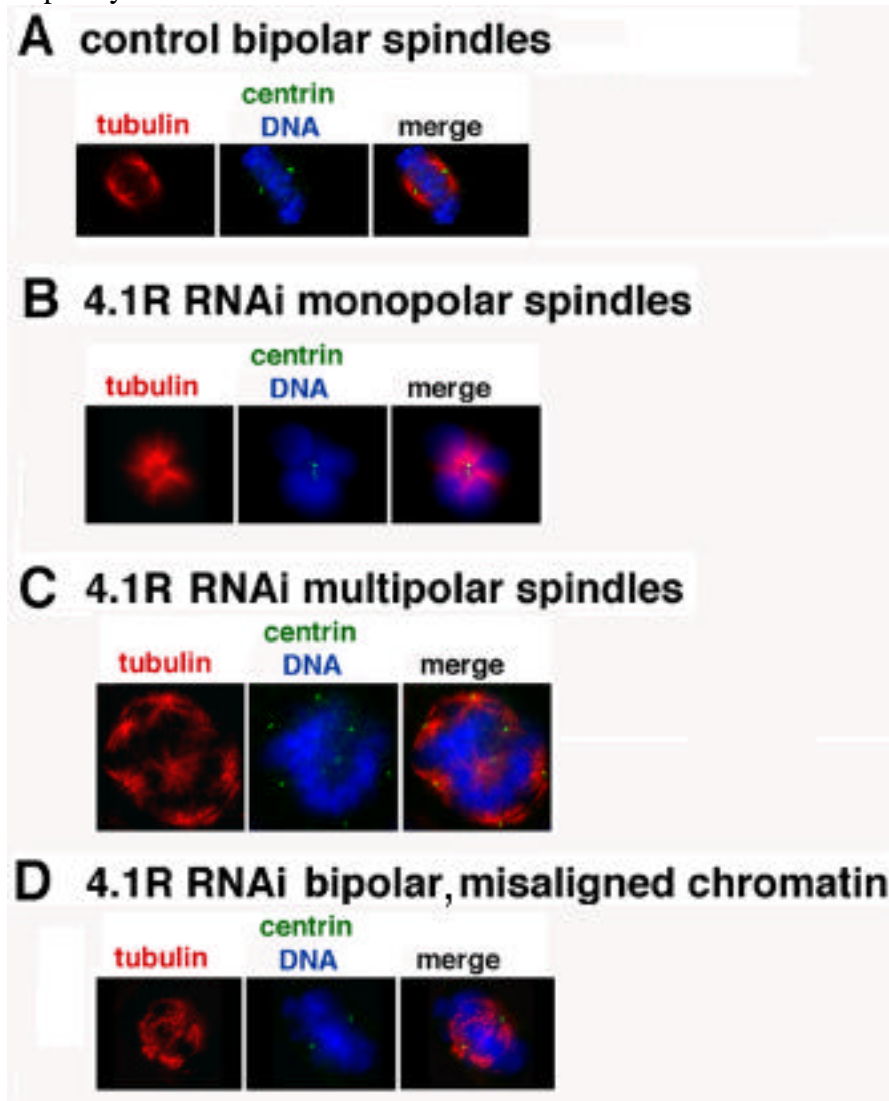


Figure 6 Mitotic spindle abnormalities after 4.1R RNAi. (A) Control mitotic spindles have microtubules converging at two opposing poles each containing focal centrin. Microtubules extend from each pole towards chromatin which forms a bisecting metaphase plate. (B-D) Mitotic cells with downregulated 4.1R expression have three main classes of mitotic spindle abnormalities. (B) Monopolar spindles with microtubules radiating towards chromatin organized in a rosette. The central polar area contains centrioles detected with anti-centrin. (C) Multipolar spindles with more than two poles marked by multiple centrin and weakly or strongly convergent

microtubules. Chromatin is relatively uncondensed. (D) Bipolar spindles with misaligned, uncondensed chromatin. Bar=5 μ .

Since centriole maturation, separation, and properly organized spindles are prerequisites for proper cytokinesis, we predicted that knockdown of 4.1R expression would produce cytokinesis defects. Indeed, 4.1R RNAi-treated anaphase cells often displayed lagging chromatin trapped between nascent daughters and inappropriately localized spindles.

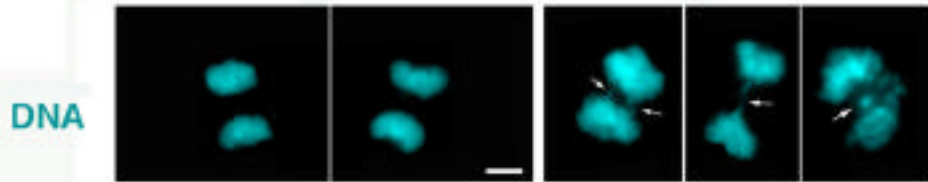


Figure 7 Cytokinesis defects after 4.1R RNAi (A) Examples of anaphase cells with lagging chromosomes (right top panel, arrows).

Finally, since abnormal centrosome function, defective mitotic spindles and aberrant cytokinesis would be predicted to impact cell cycle progression which can be altered in breast cancer cells, we compared 4.1R RNAi treated cells with control populations by flow cytometry to characterize their cell cycle patterns. Our results show that relative to controls, there were similar amounts of 4.1R depleted cells in G1, but larger amounts in S phase and lower amounts in G2/M. Therefore, downregulation of 4.1R expression does perturb cell cycle progression in a specific way. These data potentially may be relevant to changes in cell cycling observed in breast cancer cells.

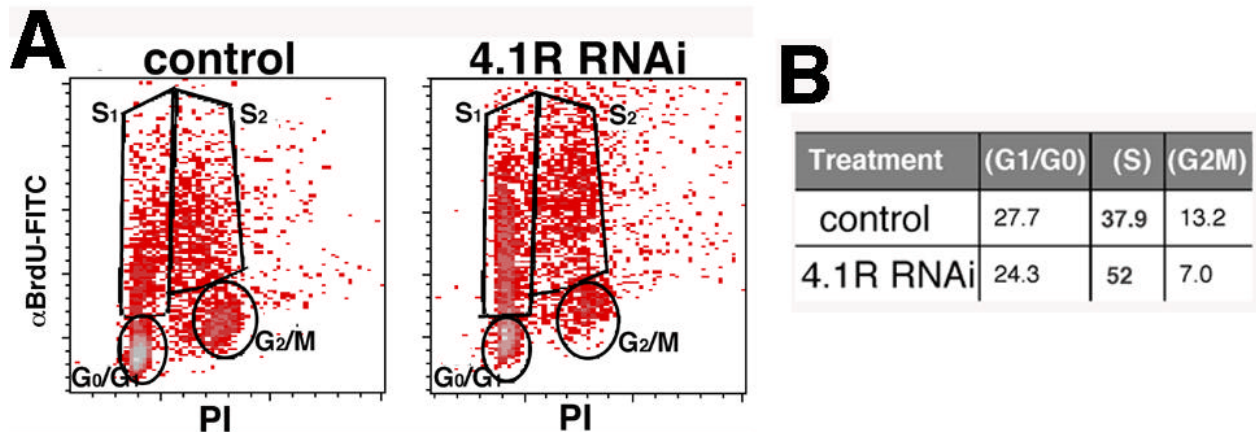


Figure 8 Cell cycle progression is altered after 4.1R RNAi treatment. (A) FACS analysis of control and 4.1R RNAi treatment. Cells synthesizing DNA were labeled with FITC-BrdU and DNA was stained with propidium iodide (PI) S1, early S phase; S2, later S phase. (B) Table of percentages of cells in various phases of the cell cycle.

These studies provide important information that key morphologic changes occurring when 4.1R expression is downregulated fall into categories that have been previously reported in breast cancer. Although we were not able to extend our work to breast cancer lines within the time frame and budget of this Concept Award, our results emphasize that this is very likely to be a feasible and worthwhile avenue of future investigation.

In sum, funding during the period of this Concept Award has enabled us to make a preliminary assessment of 4.1R distribution in several breast cancer cell lines with varying degrees of centrosome amplification and malignancy. Our observations are relevant to roles of 4.1R both in potential mechanisms of hyperamplification as well as in altered functional capacity of a subset

of amplified centrosomes which do not have associated 4.1R. In support of the latter prediction, we identified specific informative morphologic perturbations of centrosomes, spindles and anaphase cells, coupled with a definite alteration of S and G2/M phases of the cell cycle, when 4.1R expression is altered in a model human cell line. Thus, the Concept award has permitted us to initially test and validate our hypothesis. Future funding would enable us to explore the functional roles of 4.1R and other 4.1 family members in providing a mechanistic understanding of the biogenesis of centrosome and mitotic spindle abnormalities present in breast cancer cell lines and breast tumor samples that are associated with poor clinical outcomes.

KEY RESEARCH ACCOMPLISHMENTS

- **Established that 4.1R is a mature centriole component**
- **Demonstrated that 4.1R resides at the distal region of mature centrioles, an area responsible for microtubule anchoring**
- **Determined that 4.1R distribution at centrosomes varies with the cell cycle**
- **Established suitable conditions for fixation, permeabilization, immunolabeling and immunofluorescent imaging of 4.1R relative to centrosome markers in several breast cancer cell lines**
- **Found that breast cancer cell lines with normal numbers of centrosomes followed the patterns of centrosomal 4.1R localization observed in non-transformed human cells**
- **Observed that in aggressive breast cancer cell lines with amplified centrosomes, only a subset of their centrosomes contained 4.1R epitopes**
- **Identified three RNA duplexes capable of specifically downregulating 4.1R expression (RNAi) and a control duplex which did not affect 4.1R expression**
- **Determined parameters for specific silencing of 4.1R expression after transfection with 4.1R RNAi**
- **Characterized defective microtubule anchorage/organization at centrosomes after 4.1R RNAi**
- **Identified three classes of mitotic spindle pole defects after 4.1R RNAi: monopolar spindles, multipolar spindles and bipolar spindles with misaligned chromatin**
- **Examined anaphase cells after 4.1R RNAi and observed lagging chromatin between the separating daughter cells**
- **By flow cytometry, determined that 4.1R RNAi-treated populations had increased numbers of cells in S phase and decreased numbers of cells in G2/M relative to controls**

REPORTABLE OUTCOMES

Abstracts:

Krauss, S W, Go, M and Spence, JR. Roles of Protein 4.1 in Centrosome and Mitotic Spindle Aberrations in Breast Cancer Pathogenesis, Era of Hope, P29-16, p 201, 2005

Spence, JR, Go, M, Bahmanyar, S, Barth, A, Nelson, WJ, and Krauss, SW. Defective Centrosomal and Spindle Functions after Protein 4.1R Downregulation, Mol Biol of the Cell 16, #866, 365a, 2005

Presentations:

Dept Molecular & Cellular Physiology, Stanford University Medical School

Dept of Biochemistry and Molecular Biology, Pennsylvania State Medical School

Dept of Genome Biology, Lawrence Berkeley National Laboratory

Publications:

Downregulation of protein 4.1R, a mature centriole protein, impairs centrosome function, bipolar spindle organization and anaphase

Sharon Wald Krauss, Jeffrey R. Spence, Shirin Bahmanyar, Angela I. M. Barth, Minjoung M. Go, Debra Czerwinski, and Andrew J. Meyer

Molecular Biology of the Cell, 2006 (currently under revision)

Funding applied for based on this work: DOD Breast Cancer Idea Award

Personnel partially supported

Dr. Sharon Wald Krauss, P.I.

Ms. Minjoung Go

CONCLUSION

We proposed to test the novel hypothesis that protein 4.1 is of critical importance to centrosome and mitotic spindle aberrations that directly impact aspects of breast cancer pathogenesis. We characterized 4.1R, one member of the new protein 4.1 family, as a component of mature centrosomes, major microtubule organizing structures in interphasic cells. Mature centrosomes become the poles of mitotic spindles responsible for accurate segregation of duplicated chromosomes between dividing cells. Consequently, alterations in 4.1 could engender defects in functions of centrosomes, mitotic spindles and cytokinesis. We first analyzed the centrosomal distribution of 4.1R in breast cancer cell lines with normal vs hyperamplified centrosomes. We observed that 4.1R resides at only a subset of amplified centrosomes in the malignant breast cancer cell lines. Although there are several ways in which cancer cells can acquire supernumerary centrosomes, our data indicate that at least one mechanism operative in generating amplified centrosomes in these breast cancer cells is unlicensed centrosome reduplication, a process known to occur during S phase. Furthermore, the fact that 4.1R is present at only a subset of centrosomes indicates that the other supernumerary centrosomes are not fully mature and may have altered functional capacities. We next directly tested the effects of downregulating 4.1R expression. We identified specific RNA duplexes which silence 4.1R (RNAi) as well as a control duplex. After exposing cells to 4.1R RNAi, we characterized perturbed centrosomal functions, several classes of aberrant mitotic spindles, defects in cytokinesis and altered cell cycle progression. Thus our initial data strongly support our hypothesis. If funding can be obtained for future investigations to continue providing direct evidence of critical roles of 4.1R in breast cancer biogenesis, prognostic and diagnostic tests could be implemented based on assessing genetic variations as well as levels of expression of this gene in individuals. This may be as straightforward as analysis of lymphocytes isolated from patient blood samples (4.1R is expressed in lymphocytes). It will also be important to evaluate the contributions of other 4.1 family members, in particular 4.1G, to breast cancer biogenesis. An understanding of the roles of 4.1 in centrosomal and spindle abnormalities characteristic of many breast cancers can lead to identification of 4.1 or other interacting proteins as new therapeutic targets. Our approaches may also serve as an example of ways to define specific roles of other multifunctional cytoskeletal proteins in breast cancer, currently an area of much active biomedical research.

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- Krauss, S.W., C.A. Larabell, S. Lockett, P. Gascard, N. Mohandas, and J.A. Chasis. 1997a. Structural protein 4.1 in the nucleus of human cells: dynamic rearrangements during cell division. *J Cell Biol*. 137:275-289.
- Krauss, S.W., G. Lee, J.A. Chasis, N. Mohandas, and R. Heald. 2004. Two protein 4.1 domains essential for mitotic spindle and aster microtubule dynamics and organization in vitro. *J Biol Chem*. 279:27591-8.
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APPENDIX

See following pages for ERA of Hope and ASCB abstracts

ROLES OF PROTEIN 4.1 IN CENTROSOME AND MITOTIC SPINDLE ABERRATIONS IN BREAST CANCER PATHOGENESIS

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Important pathological hallmarks of many breast cancers include centrosome amplification, spindle pole defects leading to aberrant chromosome segregation, altered nucleoskeletal proteins and perturbed cytokinesis. Previously we established in human cells that protein 4.1, initially described as a human red cell skeletal protein, is also a component of centrosomes, mitotic spindles, nuclear skeleton and the midbody at cytokinesis. Importantly we recently showed by depletion/add back experiments that protein 4.1 itself is crucial for proper centrosome, spindle and nuclear assembly and that dominant/negative 4.1 peptides added to cell extracts produced multipolar and asymmetric mitotic spindles and disrupted microtubule organization necessary to assemble and maintain centrosomes. Taken together, these data lead us to hypothesize that protein 4.1 is involved in centrosome dynamics, the fidelity of cell division and in cell cycle progression.

Protein 4.1 is now known to be a multigene family. We are investigating roles of two family members, 4.1R (red cell) and 4.1G (generally expressed) in centrosome amplification and mitotic spindle aberrations in breast cancer pathogenesis. Centrosomes are composed of a cylindrical centriole pair surrounded by a larger fibrogranular area, the pericentriolar material (PCM). The more mature (or mother) centriole has “appendages” at its distal end that appear to anchor cytoplasmic microtubules. Centrosomes duplicate to become mitotic spindle poles for partitioning chromosomes.

In order to decipher 4.1 functions in breast cancer, we studied the detailed distribution of 4.1R and 4.1G at centrosomes. We report here that, by immunofluorescent microscopy, protein 4.1 R and 4.1G localize differentially within centrosomes. Protein 4.1R surrounds the centriolar “barrel” while 4.1G localizes in the outlying PCM. In mitotic spindles 4.1R is again associated with centrioles while 4.1G is distributed in the spindle matrix. Moreover, we observed that 4.1R is specifically associated with the mature (or mother) centriole, coincident with mature centriole marker proteins ninein and p150/glued by immunofluorescent microscopy. During cell cycling, 4.1R is at one centriole but spreads to the maturing daughter centriole during S phase, finally localizing during G2 at both mature centrioles after centriole duplication. Protein 4.1G epitopes remain in the PCM throughout the cell cycle. Applying these observations to breast cancer cell lines, we find that in breast cancer cell lines with normal centrosome numbers (MCF10A transformed cells and MCF7 tumor cells), protein 4.1R localizes to one or both centrioles in randomly growing populations. By contrast, in breast cancer cell lines with amplified centrosomes, such as MDAMB231 and T47D, 4.1R is observed at a subset of hyperamplified centrioles defined by centrin immunostaining. We are currently investigating 4.1R and 4.1G distribution in other breast cancer cell lines in preparation to assess changes in cellular phenotypes after specific downregulation of protein 4.1R and G. Our ultimate aim is to determine if 4.1 or its binding partners could be important chemotherapeutic targets in breast cancer treatment.

Defective Centrosomal and Spindle Functions after Protein 4.1R Downregulation

J. R. Spence,¹ M. Go,¹ S. Bahmanyar,² A. Barth,² W. J. Nelson,² S. W. Krauss¹; ¹Genome Biology, UC-LBNL, Berkeley, CA, ²Molecular and Cellular Physiology, Stanford University School of Medicine, Palo Alto, CA

Multifunctional structural proteins, serving as adaptors or linkers, are essential for cellular remodeling processes. Protein 4.1R was initially characterized as a crucial ~80kD structural component of mature red cells with well-defined functions stabilizing interactions within spectrin-actin lattices and cytoplasmic domains of transmembrane proteins. In nucleated cells, we previously characterized 4.1 as an integral “core” centrosome component, colocalizing with centriolar tubulin, in the surrounding pericentriolar matrix, and on fibers connecting centriolar pairs. As multifunctional structural proteins, centrosomal 4.1 isoforms could serve as structural elements linking components to impart dynamic properties and fidelity needed for centrosomal functions. Using an open-cell *Xenopus* extract system, we showed that 4.1 is essential for spindle and centrosome assembly and for regulation of microtubule dynamics and organization. Current investigations in mammalian cells include both 4.1R (red cell) and another family member, 4.1G (generally distributed). Using 4.1 gene-specific antibodies we observed that both 4.1R and 4.1G localize at centrosomes but have differential subcentrosomal distributions during the cell cycle. To probe 4.1R function, we specifically downregulated 4.1R expression by RNAi and characterized a unique phenotype relating to centrosomal dysfunction: (1) interphasic cells with disorganized tubulin not emanating from a centrosomal focus and multinucleated cells often containing disparate-sized nuclei; (2) perturbed ninein distribution at mature centrioles; (3) decreased frequency of mature centriole separation preceding entry into mitosis; (4) monopolar and multipolar mitotic spindles with misaligned decondensed chromatin, disorganized microtubules and mislocalized NuMA; (5) defective cytokinesis including chromosome missegregation, spindle dysmorphology, and improper tubulin bridge formation. These data suggest that 4.1R, acting as an adaptor or linker to multiple mitotic targets, could affect cell division by regulating the architectural integrity of cell division machinery. Based on our data we are analyzing 4.1 interactions impacting microtubule nucleation/anchoring, chromatin condensation and motor-dependent microtubule organization.